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13. ABSTRACT (Maximum 200) In order to evaluate the effects of ErbB2 (neo) on c-src activation, nontumorigenic human mammary epithelial cells (184.A1 line) were neo-plastically transformed with ErbB2. Transformed cells exhibited 4-6 fold higher activity of c-src, measured as phosphorylation of a substrate peptide. However, there was no detectable change in c-src content of transformed cells. The activitation of c-src was accompanied by increased phosphorylation of tyorsine 416, and by decreased phosphorylation of tyrosine 527, suggesting a kinase or phosphatase-mediated mechanism of c-src activation. The effect of ErbB2 was not mimicked by another strongly trans-forming gene, v-Ha-ras. However, the effect was observed in other nontumorigenic mammary epithelial cell lines, including the mouse line NMuMG and the human line MCF 10A. These results suggest that overexpression of ErbB2 in mammary epithelial cells directly influences c-src activity (as opposed to altering stromal interactions or systemic factors, such as hormone levels.) Furthmore, they suggest a possible role of c-src activation in ErbB2 tumorigenesis or tumor progression.

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INTRODUCTION

The hypothesis of this proposal is that ErbB2 expression regulates the activity of c-src and that this activation is a factor in mediating ErbB2 induced tumorigenesis. Previous work with human tumors has shown increased c-src activity (Ottenhoff-Klaff et al., 1992). Tissue from mice overexpressing ErbB2 show increased activity of c-src (Muthuswamy et al., 1994). Knockout mice lacking c-src also exhibit decreased tumorigenesis induced by polyomavirus middle T antigen (Guy et al., 1994). Data from our laboratory and others (Zhair et al., 1993) show that non-tumorigenic mammary epithelial cells are readily transformed to a tumorigenic phenotype by overexpression of ErbB2. This tumorigenic transformation is accompanied by increased activity of c-src (see below).

The overall project has two major areas of focus: the mechanism by which ErbB2 activates c-src and the consequences of c-src activation. The first year's work has focused on the activation of c-src by ErbB2. There are several ways by which ErbB2 could regulate c-src activity: by altering c-src expression levels, by decreasing expression and/or activity of csk, by increasing expression and/or activity of c-src-directed phosphotyrosine phosphatases or by phosphorylation-independent activation of c-src.

As described in the STATEMENT OF WORK, year 1 consisted of verifying ErbB2 activation of c-src in our model cell lines, and mapping phosphorylation sites of c-src. This consists of:

    A.   Determining the effect of ErbB2 overexpression on src activity

    B.   Determining the effect of ErbB2 overexpression on src phosphorylation

    C.   Determine the effect of ErbB2 overexpression on src expression

    D.   Determining if src activation by ErbB2 is induced by oncogenes other than ErbB2

    E.   Determining if other src-related kinases are activated or altered by ErbB2

BODY

Experimental Methods

Cell Lines

The nontumorigenic human mammary epithelial cell line 184.A1 was used in most studies covered by this report. Cells were routinely grown in DMEM:F12 + 10% FBS, 5 µg/ml insulin and 10 ng/ml EGF).

Cells were transfected by ErbB2 and with v-Ha-ras in vectors containing a G418 resistance marker. Vectors were cloned in DH5α E. coli using standard procedures. Cells (5×10^6 in 0.5 ml HBSS) were placed in electroporation cuvets with 2 mm electrode space and pulsed with 1.2 kV/cm field strength. Cells were left on ice for 10 minutes, then returned to culture media. After 24 hours, media was changed to contain 400 µg/ml G418 sulfate and selection continued for 4 weeks.

Characteristics of cell lines

ErbB2 expression

To assess overxpression of ErbB2, cells were plated in 60 mm culture dishes (10^7 cells per dish and grown for 24 hours. Media was removed and SDS loading buffer lacking mercaptoethanol and bromophenol blue added to plates. Cells were scraped into tubes and heated to 95°C for 5 minutes. Protein content was then assessed by BCA assay (Pierce Chemical Co., Rockford, IL) and equalized among samples. Bromophenol blue and 2-mercaptoethanol were added to samples, equal protein was then separated by SDS-PAGE using a 7.5 % separating gel (Laemmli, 1970) and transferred to PVDF membranes (Towbin et al., 1979; Fenton and Sheffield, 1993). Membranes were probed with anti-ErbB2 (Transduction Laboratories, Lexington, KY) and detected with chemiluminescence (DuPont, Boston, MA) as described previously for other proteins (Fenton and Sheffield, 1993). Band intensity was quantitated by computer assisted densitometry (Collage®, Fotodyne, New Berlin, WI).

Growth on soft agar

Cells (10^4) were suspended in 2 ml of 0.3% agarose dissolved in culture media and layered onto 2 ml of hardened 0.5% agarose in culture media. After 10 days, cells were observed for growth.

Growth in nude mice

Inguinal (fourth) mammae of 3 week old athymic mice were exposed and epithelial containing portions excised. Cell suspension (10 µl of a suspension of 1×10^8 cells/ml in HBSS) were injected into the resulting fat ape and incisions closed with wound clips. Mice were observed daily for tumor formation and euthanized with 5 mg pentobarbital i.p. when tumors were palpable. Tumors were processed for histological evaluation by fixing in phosphate buffered formalin, embedding in plastic, cutting into 5 µm thick slices, mounting on slides and staining with hematoxylin and eosin.

Measurement of c-src activity

Cells were lysed with lysis buffer (30 mM sodium pyrophosphate, 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% BSA, 1 mM sodium orthovanadate, 1 mM PMSF). Lysates were clarified by centrifugation for 10 minutes at 15,000 g, protein content determined by BCA assay and protein equalized among samples. Samples were then incubated for 2 hours at room temperature with anti c-src and agarose conjugated protein A and G. Samples were centrifuged and beads washed 4 times with lysis buffer. Beads were then incubated with kinase buffer (10 µl of 200 mM HEPES, pH 7.0 containing 125 mM $MgCl_2$, 25 mM $MnCl_2$ and 0.25 mM sodium orthovanadium. Substrate (5 µl of a 0.5 mM solution of [lys^{19}]cdc2(6-20) was added and reactions started by adding 5 µl of γ^{32}P-ATP (0.5 mM, specific activity of 5,000 dpm/pmole). After 5 minutes, reactions were stopped by adding 10 µl of 50% acetic acid and samples were centrifuged (5,000 g for 5 minutes). Supernatant was spotted onto Whattman P81 phosphocellulose paper, washed 4 times with cold 100 mM phosphoric acid, rinsed with acetone, dried and counted by liquid scintillation. In addition, reactions were performed without peptide or

with [val^{12}ser^{14}lys^{19}]cdc2(6-20) (which should not be phosphorylated by src) as a substrate. c-src content of immunoprecipitates was determined by western blot analysis, essentially as described above for ErbB2 and resulting data used to correct for any differences in src content.

Measurement of c-src phosphorylation

C-src was immunoprecipitated as described above. Src was then digested with 50 mg/ml cyanogen bromide in formic acid and lyophylized. Resulting peptides were separated by tricine PAGE (van der Geer et al., 1993) and transferred to PVDF membranes by electroblotting. Membranes were then blocked and probed with anti-phosphotyrosine as previously described (Fenton and Sheffield, 1993). Density of bands was determined by densitometry (Collage®).

Measurement of c-src Expression

Cells were lysed as described above for ErbB2 expression. Protein content of lysates was equalized, proteins separated by SDS-PAGE (12% separating gel) and probed with anti-c-src as above.

Measurement of other src-related kinases

Other members of the src family, including lyk, lyn and fyn, were immunoprecipitated as described for c-src and activity estimated essentially as described for c-src.

Results

Characterization of cell lines

ErbB2 expression

Cells transfected with ErbB2 exhibited substantially greater concentrations of ErbB2 than parental cells (Figure 1), indicating that the ErbB2 transfected lines dramatically overexpressed the gene at the level of cell protein content.
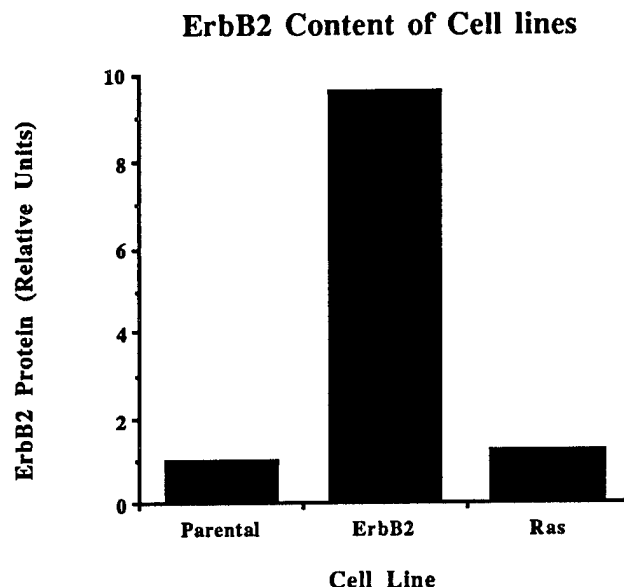
ErbB2 Content of Cell lines

Figure 1. ErbB2 content of parental 184.A1 cells and cells transformed with ErbB2 and v-Ha-ras. Mean of 3 determinations.

Growth on soft agar

ErbB2 and ras transformed cells grew readily on soft agar, whereas the parental cells were incapable of growing on soft agar. This, together with nude mouse tumor formation (below) suggests that the cells are tumorigenically transformed.

Growth in nude mice

ErbB2 and ras transformed cells were capable of forming tumors in nude mice (100% of inoculated mice had palpable tumors within 30 days). The tumors were typical of poorly differentiated breast adenocarcinomas. No tumors were formed by the parental cells, suggesting that ErbB2 and ras induced tumorigenic transformation.

c-src activity

Src activity (Figure 2) was dramatically increased by ErbB2 transformation, but only modestly increased by ras transformation. When corrected for amount of c-src in immunoprecipitates, essentially identical results were obtained, as c-src content of immunoprecipitates exhibited little difference among treatments (Figure 3). This would be

consistent with a model in which ErbB2 induces c-src activity by activating existing enzyme, rather than inducing c-src expression.
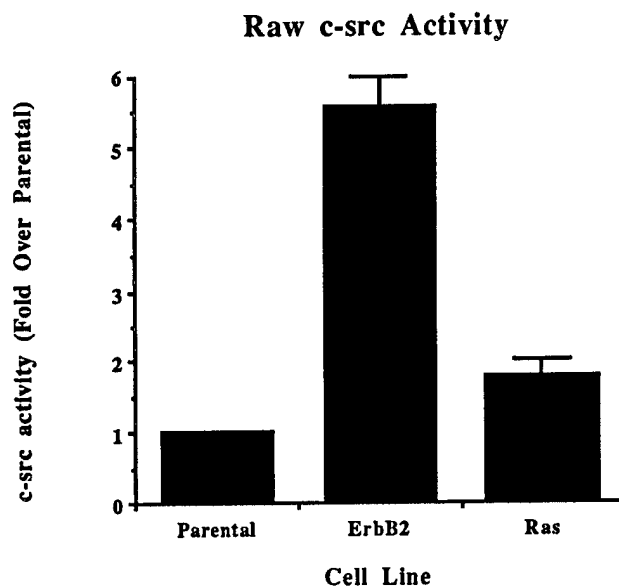
Raw c-src Activity

Figure 2. Effect of transformation by ErbB2 or Ras on total c-src activity in 184.A cells. Mean ± SEM of 4 determinations.
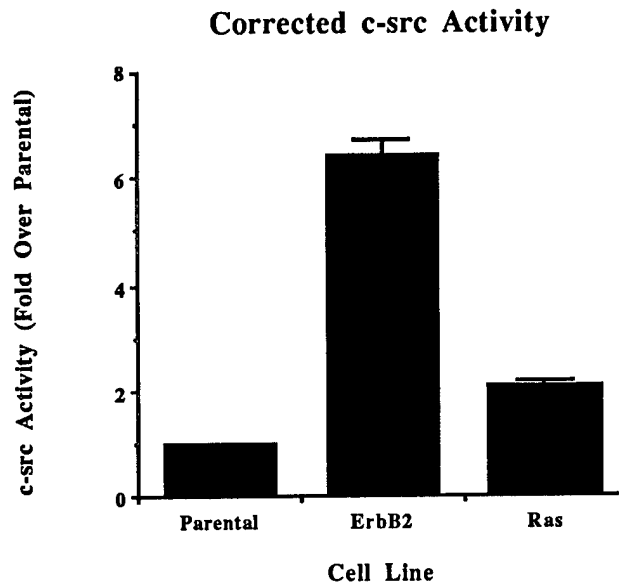
Corrected c-src Activity

Figure 3. c-src activity in 184.A1 cells or 184.A1 cells transfected with ErbB2 or ras. Values reported after division by relative amount of c-src in the immunoprecipitate in order to give a specific activity change. Mean ± SEM of 4 observations.

Linearity of assay over time was verified by conducting assays for 2, 4, 6 or 8 minutes (Figure 4) Results indicated that assay was linear over time.

Linearity of Kinase Assay

$y = 0.75000 + 2.0800x \quad R^2 = 0.988$

● Parental
○ ErbB2

$y = 0.15000 + 0.45000x \quad R^2 = 0.967$

c-src Activity (pmole PO4 incorporation)
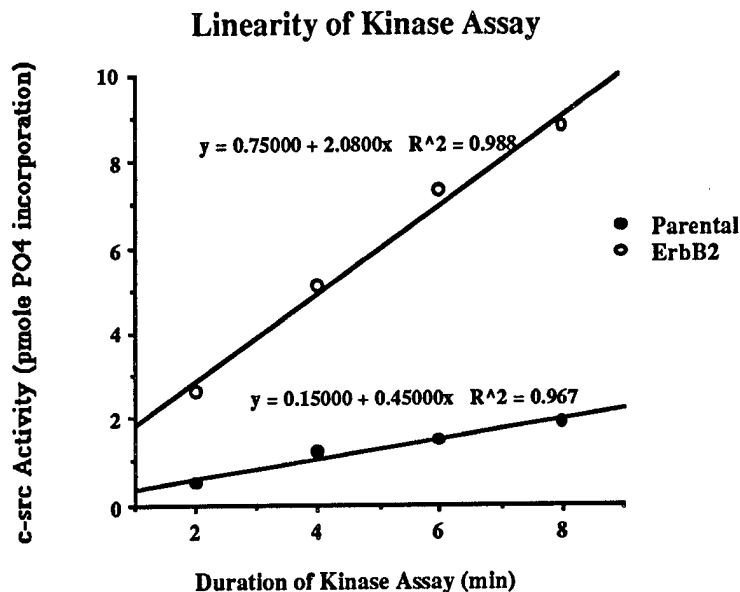
Duration of Kinase Assay (min)

Figure 4. Linearity over assay time of c-src kinase assay on parental and ErbB2 transformed 184.A1 cells.

The c-src kinase assay used above was found to be dependent of added substrate peptide (Figure 5). Omission of the peptide or use of a peptide not phosphorylated by c-src yielded essentially background activity, as did omission of the enzyme.
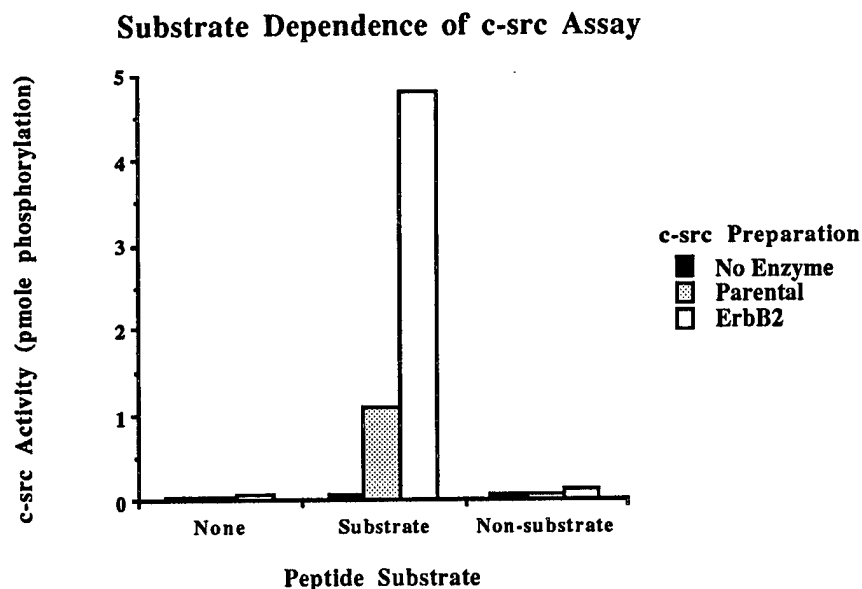
Substrate Dependence of c-src Assay

c-src Activity (pmole phosphorylation)

c-src Preparation
■ No Enzyme
▨ Parental
□ ErbB2

None   Substrate   Non-substrate

Peptide Substrate

Figure 5. Effect of omission of enzyme, omission of substrate or substitution of a non-substrate peptide ([val^{12}ser^{14}lys^{19}]cdc2(6-20)) on c-src activity. Mean of 2 determinations.

Diluting the enzyme preparation used in kinase assays (Figure 6) caused a corresponding decrease in kinase activity, indicating that the results were linear over the enzyme concentration range used in the studies.
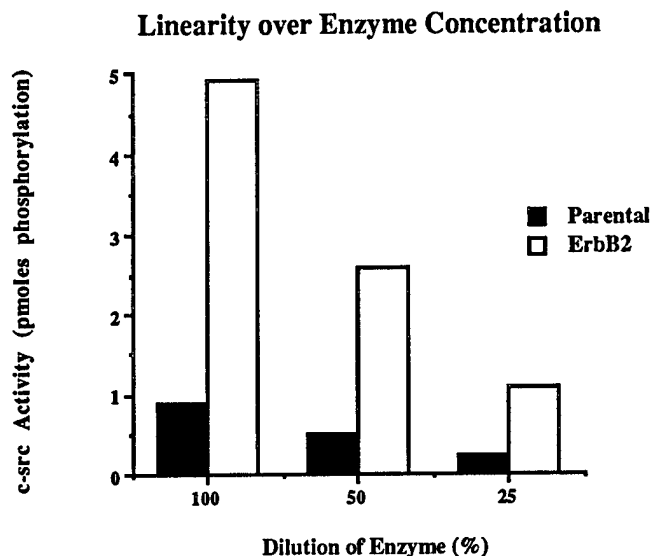
Linearity over Enzyme Concentration

Figure 6. Effect of diluting enzyme preparation (immunoprecipitate) on measured c-src kinase activity. Mean of 2 experiments.

Performing the assay using different starting concentrations of substrate peptide indicated that the results obtained were not an artifact of substrate concentration (Figure 7).
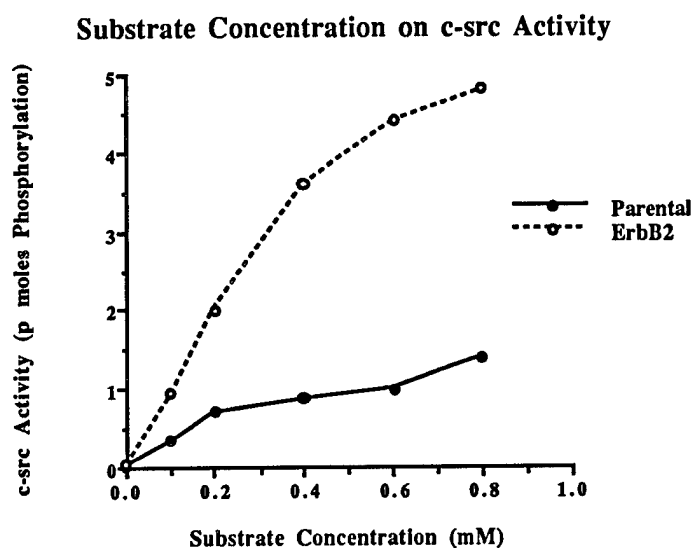
Substrate Concentration on c-src Activity

Figure 7. Dilution of substrate peptide on c-src kinase activity estimates in 184.A1 cells and cells transformed with ErbB2. Mean of 2 determinations.

ATP concentration curve (Figure 8) indicated that kinase assays were conducted at near-maximum concentrations of ATP, and that ATP depletion is not likely to be a factor in the results obtained.

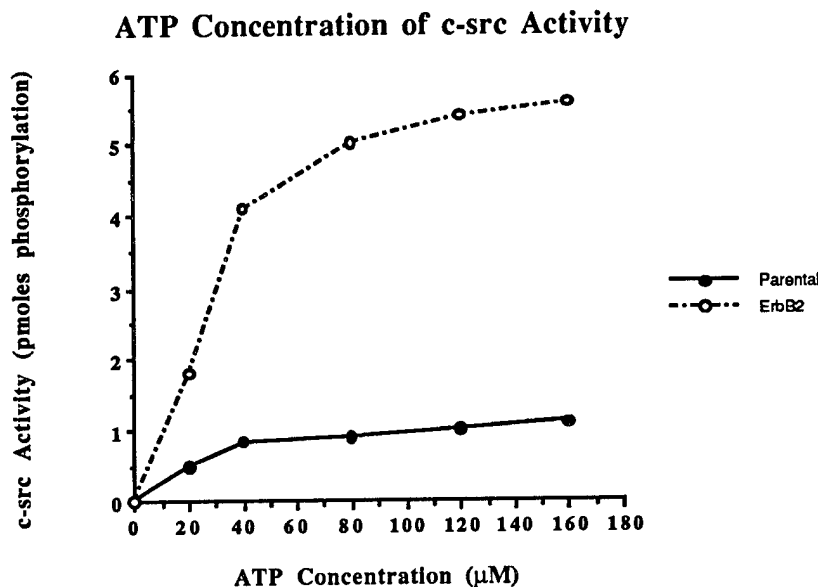**ATP Concentration of c-src Activity**

Figure 8. Effect of varying ATP concentration on c-src kinase activity estimates in 184.A1 cells and 184.A1 cells transformed with ErbB2. Mean of 2 determinations.

c-src phosphorylation

Peptide mapping indicated a readily-detectable (approximately 3-4 fold) increase in tyrosine phosphorylation of a 10 kDa cyanogen bromide peptide, corresponding to the autophosphorylation site of c-src, and a corresponding decrease in tyrosine phosphorylation of a 4 kDa peptide (Figure 9), which corresponds to the inhibitory tyrosine at position 527 (Nada et al., 1993).
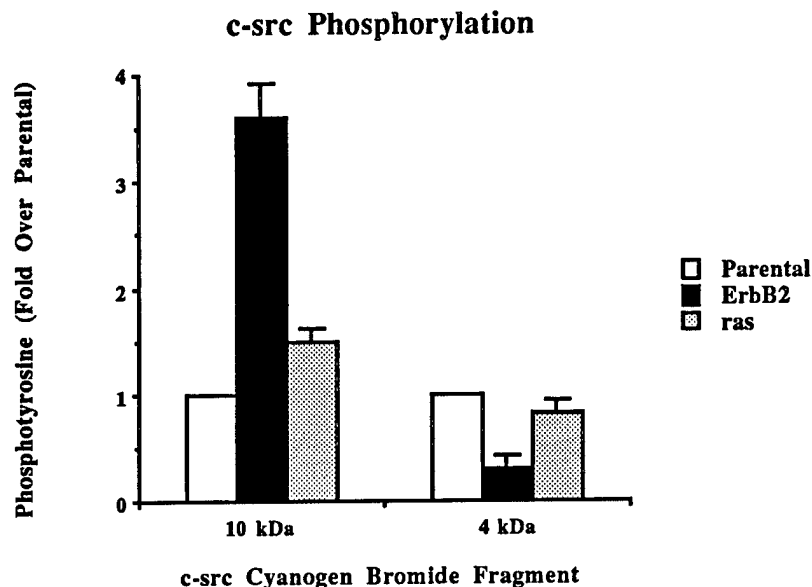
c-src Phosphorylation

Figure 9. Phosphorylation site of c-src in vivo in 184.A1 cells and cells transfected with ErbB2 or ras. Mean ± SEM of 3 determinations.

c-src Expression

Total c-src content of 184.A1 cells, ErbB2 transformed cells and ras transformed cells was similar, indicating that changes observed above were likely to be due to activation of existing c-src, not increased c-src expression (Figure 10).
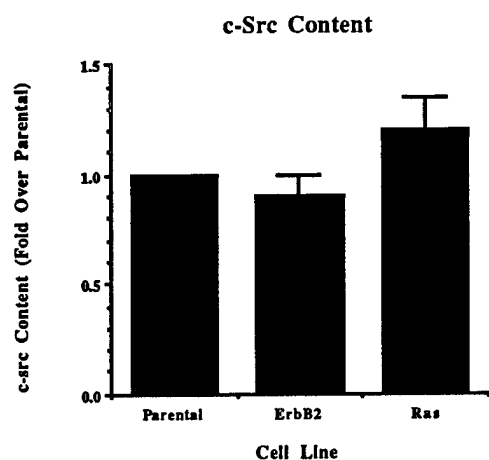
c-Src Content

Figure 10. Effect of ErbB2 or ras transformation of 184.A1 cells on c-src content, as determined by Western blot analysis. Mean ± SEM of 4 determinations.

Other src-related kinases

To date, we have not observed activation of lyk, lyn or fyn in response to ErbB2 transformation.

Other Cell Lines

Most of the work to date has been on 184.A1 cells and their transformed derivatives. However, we have also transformed other cell lines with ErbB2 (some in preliminary studies described in the grant proposal). These include NMuMG (a nontumorigenic mouse mammary epithelial line) and MCF 10A (a non-tumorigenic human mammary epithelial cell line). In both of these lines, ErbB2 increases c-src activity with little or no change in c-src protein content of cells. Although baseline levels of c-src activity vary somewhat among cell lines, the fold induction by ErbB2 was similar among lines (Figure 11).
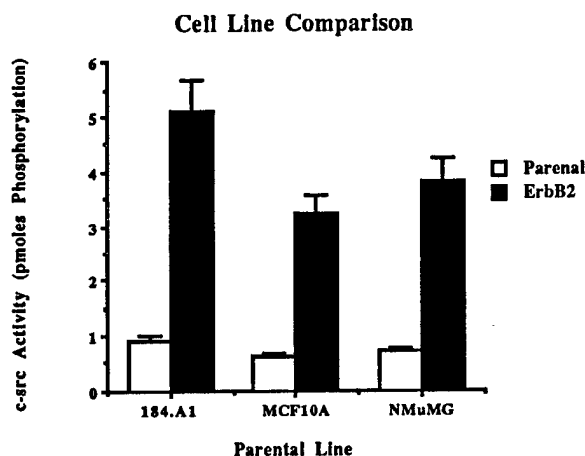
Cell Line Comparison

Figure 11. Activation of c-src by ErbB2 transformation in various cell lines. Mean ± SEM of 3 determinations.

Discussion

Results of these studies largely confirm previous (unpublished) studies in our laboratory that mouse mammary epithelial cells (NMuMG) when transfected with ErbB2. These studies indicate that ErbB2 does not dramatically alter the amount of c-src in cells, but does increase c-src activity 4-6 fold. This appears to be associated with increased in vivo tyrosine phosphorylation on the autophosphorylation site of c-src (tyrosine 416) and decreased tyrosine 527 phosphorylation. This would appear to represent a mechanism of activation of c-src (Cooper and Howell, 1993).

There are several possible mechanisms by which ErbB2 could activate c-src. ErbB2 could decrease expression or activity of the kinase responsible for phosphorylating tyrosine 527 (most likely csk or c-terminal src kinase, Cooper and Howell, 1993). Alternatively, ErbB2 could increase the activity or level of phosphotyrosine phosphatases directed toward the C-terminus of c-src. These hypotheses are the subject of work for year 2 of the project.

Recommendations

Based on the results of Year 1, the plan of work outlined for year 2 remains plausible. This includes determining the effects of ErbB2 on expression and activity of csk and src-directed phosphatases. We envision no major modifications to those plans at this time.

CONCLUSIONS

Results to date indicate that overexpression of ErbB2 in nontumorigenic mammary epithelial cells increases activity, but not expression, of c-src. Furthermore, this effect is not mimicked by other transforming oncogenes. These results suggest that previous results in transgenic animals are likely to be due to direct effects on mammary epitheliaum, not to alterations in systemic physiology (such as hormone levels) or to altered epithelial stromal interactions. The results also suggest a likely involvement of either csk or src-directed phosphatases in this activation. Examination of the mechanism of activation is the focus of ongoing work. These results suggest that strategies to modify src activation may be useful in the development of breast cancer therapies.

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APPENDICES

None